

Population Dynamics Following the Last Glacial Maximum in Two Sympatric Lizards in Northern China

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Abstract Phylogeographic studies of *Eremias* lizards (Lacertidae) in East Asia have been limited, and the impact of major climatic events on their population dynamics remains poorly known. This study aimed to investigate population histories and refugia during the Last Glacial Maximum of two sympatric *Eremias* lizards (*E. argus* and *E. brenchleyi*) inhabiting northern China. We sequenced partial mitochondrial DNA from the *ND4* gene for 128 individuals of *E. argus* from nine localities, and 46 individuals of *E. brenchleyi* from five localities. Forty-four *ND4* haplotypes were determined from *E. argus* samples, and 33 from *E. brenchleyi* samples. Population expansion events began about 0.0044 Ma in *E. argus*, and 0.031 Ma in *E. brenchleyi*. The demographic history of *E. brenchleyi* indicates a long-lasting population decline since the most recent common ancestor, while that of *E. argus* indicates a continuous population growth. Among-population structure was significant in both species, and there were multiple refugia across their range. Intermittent gene flow occurred among expanded populations across multiple refugia during warmer phases of the glacial period, and this may explain why the effective population size has remained relatively stable in *E. brenchleyi* and grown in *E. argus*.

Keywords Lacertidae, *Eremias* lizards, mitochondrial DNA, historical demography, multiple refugia, Last Glacial Maximum

1. Introduction

Historic climatic change (e.g., climatic oscillations during the Pleistocene Epoch) affected the historical demography of extant species (Hewitt, 2000, 2004). Glacial and interglacial periods consisted of a series of alternating warming periods of limited duration (interstadials) and short-term cooling periods (stadials) (Martrat *et al.*, 2004). The effective population size of a species may decrease during the glacial period and increase during the interglacial period. However, if there were multiple

refugia within its range, the effective population size of the species might remain stable or grow during the glacial period due to intermittent gene flow between refugia during warmer periods (interstadial) (Li *et al.*, 2009; Ruiz-González *et al.*, 2013). Climatic changes during Pleistocene cycles have played a key role in shaping genetic diversity of species and left genetic signatures in present populations (Hewitt, 1996, 1999, 2000, 2004; Avise, 2000) that can help identify glacial refugia, post-glacial migration routes and population dynamics (Newton *et al.*, 1999; Petit *et al.*, 2005; Li *et al.*, 2009; Tian *et al.*, 2009; Ruiz-González *et al.*, 2013). Relative to birds and mammals, reptiles are less able to disperse and are therefore more sensitive to climatic fluctuations, making them more suitable biogeographical indicators (Camargo *et al.*, 2010; Malhotra *et al.*, 2011).

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Lacertid lizards of the genus *Eremias* are widely distributed in Southeast Europe, West and Central Asia, and Mongolia, northern China, Korea and Russia (regions of Lake Baikal) in East Asia. Of some 30 extant species, eight can be found in China, six of which (*E. argus*, *E. arguta*, *E. brenchleyi*, *E. grammica*, *E. velox* and *E. vermiculata*) are oviparous, and two (*E. multiocellata* and *E. przewalskii*) are viviparous (Zhao, 1999). The ancestor of *Eremias* lizards has been hypothesized to originate from Europe and dispersed northwards to Central Asia through Africa during the late Miocene 8–10 million years ago (Ma) (Fu, 1998; Arnold *et al.*, 2007; Mayer and Pavlicev, 2007; Hipsley *et al.*, 2009). According to this hypothesis, *Eremias* lizards found in East Asia should spread from west (Central Asia) to east, not following westward, southward or northward adaptive radiation of species found in other parts of the world (Forcina *et al.*, 2012; Mori *et al.*, 2013; Zink *et al.*, 2013). During the Last Glacial Maximum (LGM), multiple glacial refugia were available to East Asian species throughout their ranges (Qian and Ricklefs, 2000; Li *et al.*, 2009; Tian *et al.*, 2009; Bai *et al.*, 2010) primarily due to a relatively mild Pleistocene climate in that region (Weaver *et al.*, 1998; Ju *et al.*, 2007). However, as phylogeographic studies of *Eremias* lizards in East Asia have been limited, the impact of major climatic events on their population dynamics remains poorly known.

Here, we study two oviparous *Eremias* lizards (*E. argus* and *E. brenchleyi*) sympatric throughout the distribution of *E. brenchleyi* in the central and northern parts of China (Chen, 1991). The two species can coexist primarily because they use different microhabitats (*E. argus* mainly inhabits plains and hills, while *E. brenchleyi* inhabits hills only) not because they differ greatly along the resource axes of diet and time (Zhao, 1999). Both species are relatively common and have more intact population genetic structure than other species or populations that have become threatened or endangered in China (Elderkin *et al.*, 2007; Lin *et al.*, 2010; Zhao *et al.*, 2011), thus providing ideal model systems in which to investigate population histories, glacial refugia and post-glacial migration routes. Here, we used partial mtDNA sequences from the gene *ND4* to address three questions regarding population dynamics following LGM in these two species. (1) Did they survive in multiple refugia across their species range or in a single refuge during the LGM? (2) What was the pattern of recolonization routes? (3) How did effective population size vary during the LGM?

2. Materials and Methods

2.1 Sampling We sequenced the mitochondrial *ND4* gene from 128 individuals of *E. argus* from nine sites (Figure 1 A, Table 1) and 46 individuals of *E. brenchleyi* from five sites (Figure 1 B, Table 1). The most distal 2–3 mm of the tail tip from each lizard was stored in 95% ethanol. Lizards were released at their site of capture immediately after tissue sampling. The influence of tail breaks at the distal portion of the tail often is not significant in lizard species with caudal autotomy, including *Eremias* lizards (Lin and Ji, 2005; Lin *et al.*, 2006; Zhao *et al.*, 2008; Sun *et al.*, 2009; Ding *et al.*, 2012). Voucher specimens were deposited at Nanjing Normal University and assigned voucher numbers identified by locality-haplotype numbers. The sampling code, geographic information and genetic analysis are given in Appendix A, Appendix B and Table 1. We used all sequences from this study and three sequences from two lacertid lizards (*Lacerta viridis viridis*: NC_008328; *Timon lepidus*: DQ902321 and DQ902322) as ingroups, and two sequences (DQ150379 and DQ150376) from *Psammotromus algirus* (Hipsley *et al.*, 2009) as outgroups for the phylogenetic analysis based on *ND4* haplotypes.

2.2 Molecular data Genomic DNA was isolated from tail samples using standard phenol-chloroform extraction methods (Palumbi, 1996). Fragments of complete *ND4* gene from the mitochondrial genome were amplified with the primers *ND4* (5'-CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA GC-3') and leu (5'-CAT TAC TTT TAC TTG GAT TTG CAC CA-3') (Busack *et al.*, 2005). PCRs were performed in 100 µl volumes, using a hot start method; PCR cycling parameters were 94 °C for 7 min; 40 cycles of 94 °C for 40 s, 46 °C for 30 s, 72 °C for 1 min; and a final elongation step of 72 °C for 7 min. PCR products were then purified with a spin column containing Sepacry S-400 (Amersham Bioscience AB, Uppsala, Sweden) and sequenced using both forward and reverse primers on an ABI-PRISM™ 310 Genetic Analyzer (Applied Biosystems Information, USA). Sequences were edited and aligned manually using BIOEDIT version 7.0.9.0 (Hall, 1999), and were translated to amino acids with Mega 6.0 to verify if a functional mitochondrial DNA sequence was obtained and that nuclear pseudogenes were not amplified (Tamura *et al.*, 2013). All sequences were deposited in GenBank under accession numbers JN561171–JN561247.

2.3 Phylogenetic analysis Phylogenetic analysis were carried out by Bayesian inference (BI) using MrBayes

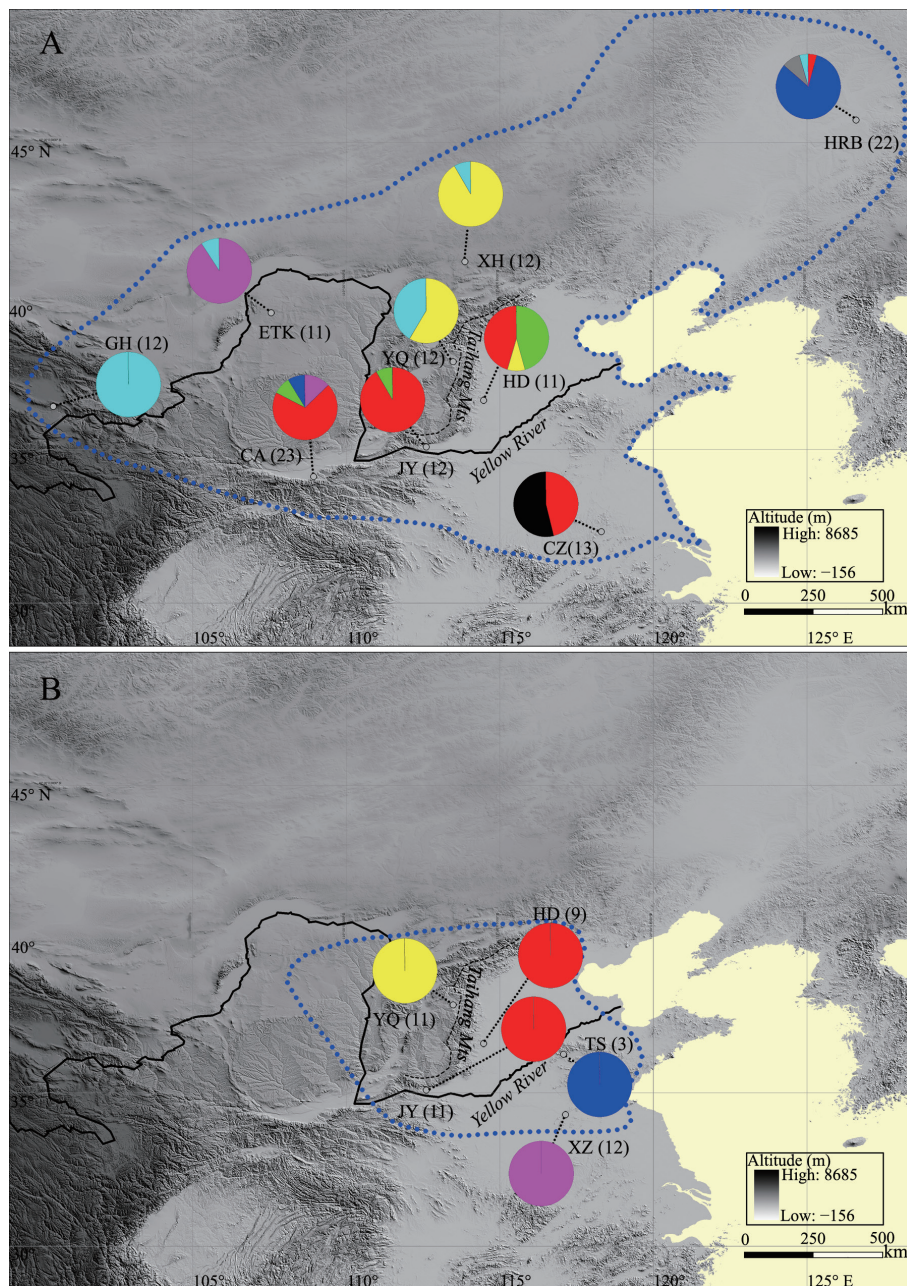


Figure 1 Map of northern China showing localities where individual *E. argus* (A) and *E. brenchleyi* (B) were collected. See text and Table 1 for sample locality abbreviations. Numbers in parentheses are sample sizes. Small circles represent sampling localities, and pie charts represent haplotype group frequencies within each locality. The blue, dotted lines outline the species range in China.

3.1.2 (Ronquist and Huelsenbeck, 2003) and MrModeltest 2.3 (Nylander, 2004). The best-fit model (GTR + I) for 1st codon position, (HKY + G) for 2nd codon position, and (GTR + G) for 3rd codon position were selected by Akaike Information Criterion (AIC) in MrModeltest 2.3. Four Markov Chains Monte Carlo (MCMC) samples were run for 5×10^6 iterations. Two independent runs were carried out to allow additional confirmation of the convergence of MCMC runs. Trees were sampled

every 500 iterations, providing 2×10^4 samples from the two runs. The first 4% of the iterations were discarded as burn-in, leaving 1.96×10^4 samples to estimate the consensus tree and the Bayesian posterior probabilities. Haplotypes with lineages were evaluated using median-joining networks (MJNs) in NETWORK 4.5.1.0 (Bandelt *et al.*, 1999).

2.4 Divergence times We used Bayesian MCMC method to evaluate divergence times and evolutionary rates. For

Table 1 Descriptive statistics by sampling locality for the two lizards studied. NH: number of haplotypes; LSH: locality-specific haplotypes; HD: haplotype diversity; ND: nucleotide diversity; D : Tajima's D ; Fu's F_s : statistics of Fu's F_s test (1997); OMMD: observed modality of mismatch distribution; SSD: the sum of square deviation between the observed and simulated mismatch distributions; Raggedness: raggedness index.

Species	Sampling locality (abbreviation)	Latitude	Longitude	n	NH	LSH	HD (h)	ND (π)	D	Fu's F_s	OMMD	SSD	Raggedness
<i>E. argus</i>				128	44		0.952	0.025	-0.59	-2.83	Multimodal	0.00725	0.01277*
	Harbin (HRB)	45.7	126.6	22	7	4	0.541	0.01	-0.97	3.1	Multimodal	0.34002***	0.12777
	Xinghe (XH)	41.1	113.9	12	4	2	0.697	0.004	-1.56	1.48	Multimodal	0.06753	0.17034
	Etuoke (ETK)	39.5	107.5	11	8	8	0.891	0.01	-2.08***	-0.59	Multimodal	0.01838	0.04298
	Yangquan (YQ)	37.9	113.6	12	6	6	0.818	0.009	1.43	1.4	Multimodal	0.13748*	0.21212
	Handan (HD)	36.6	114.5	11	6	6	0.873	0.0142	0.52	2.5	Multimodal	0.11861**	0.13058
	Gonghe (GH)	36.4	100.5	12	2	0	0.546	0.0008	1.49	1.2	Unimodal	0.03392	0.30579
	Jiyuan (JY)	35.1	112.6	12	7	7	0.909	0.008	-1.64*	0.1	Multimodal	0.28364**	0.04454
	Chang'an (CA)	34	108.9	23	6	4	0.573	0.017	-0.44	8.38	Multimodal	0.38288***	0.20803
	Chuzhou (CZ)	32.3	118.3	13	3	3	0.603	0.018	2.87	11.06	Unimodal	0.27213	0.45348
<i>E. brenchleyi</i>				46	33		0.984	0.059	1.69	-0.88	Multimodal	0.02235	0.00581
	Yangquan (YQ)	37.9	113.6	11	8	8	0.946	0.008	0.04	-1.38	Multimodal	0.01076	0.0281
	Handan (HD)	36.6	114.5	9	6	6	0.889	0.018	1.66	1.96	Multimodal	0.09925**	0.18827
	Taishan (TS)	36.3	117.1	3	3	3	1	0.004	0	-0.34	Unimodal	0.23127	0.66667
	Jiyuan (JY)	35.1	112.6	11	9	9	0.946	0.004	0.78	-5.52***	Unimodal	0.00411	0.0519
	Xuzhou (XZ)	34.3	117.2	12	7	7	0.894	0.003	-0.66	-2.68*	Unimodal	0.06078	0.24174

*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

E. argus, we set the mean of the prior distribution for the time separating the ingroup root from the present (rttm) to 45.4 Ma estimated based on the age (44.1–46.7 Ma with a mean of 45.4 Ma; Appendix A, node 5 (Hipsley *et al.*, 2009) of the split between *Eremias* and *Timon*. The standard deviation of the prior distribution for the time separating the ingroup root from the present (rtmsd) was set at one-half of rttm. The mean and standard deviation of prior distribution for rate at root node (rrate and rratesd) were set to 0.008 2 substitutions per site per 1 Ma, with the rrate value calculated by dividing the median distance between the ingroup root and the tips by rttm; the mean and standard deviation of prior for brownian motion constant nu (brownmean and brownsd) both were set to 0.022, so that $rttm \times brownmean = 1$, following recommendations accompanying the software. All remaining parameters were set to default values. In this study, the fossil calibration point was defined to the most recent common ancestor of *E. argus* according to the Middle Pleistocene (0.438–0.548 Ma) found in Chinese fossil layers (Li *et al.*, 2004) (node N3 in Figure 2). The Bayesian analysis was run for 5×10^6 generations, with a sample frequency of 100 after a burn-in of 5×10^4 generations.

To estimate intraspecies divergence time of *E. brenchleyi*, we set rttm to posterior distribution of the divergence time of *Eremias* and *Timon*, which came from the above results of *E. argus* intraspecies divergence-time

estimation rttmsd was set to equal rttm. The rrate value was calculated by dividing the median distance between the ingroup root and the tips by rttm and the rratesd value was set to equal rrate. We set all the remaining parameters to default values. We used two calibration points in these estimates (N15 and N16 in Figure 3). The N16 calibration point was based on the fossil record of ancestor of *E. argus* from the Middle Pleistocene (0.438–0.548 Ma) (Li *et al.*, 2004). The lower bound for the age of node N16 was 0.438 Ma, and the upper bound for the age of node N16 was 0.548 Ma. N15 was not a fossil calibration but a secondary calibration point from the above results of the 95% confidence interval of *E. argus* intraspecies divergence-time estimation. The Bayesian analysis was run for 5×10^6 generations, with a sample frequency of 100 after a burn-in of 5×10^4 generations.

2.5 Population genetic analyses We followed Zhao *et al.* (2011) to calculate haplotype diversity (h), mean nucleotide diversity (π), mismatch distributions, Fu's F_s and Tajima's D for each sampling site and for each species as a whole (1000 replicates). Nucleotide diversity and haplotype diversity were tested for a significant correlation with latitude using linear regression. We performed mismatch-distribution tests for goodness-of-fit against a null model of sudden population expansion calculated using SSD between observed and expected

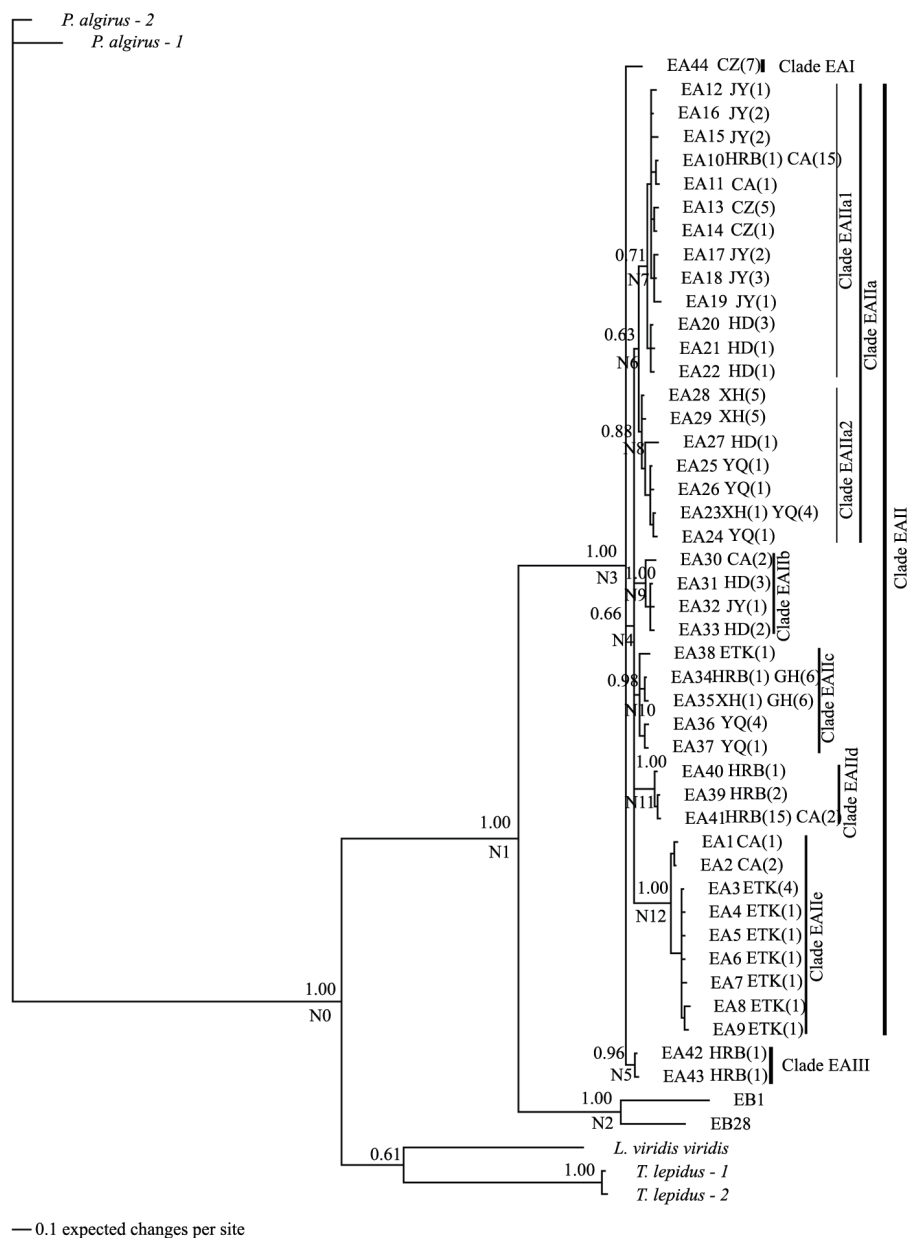


Figure 2 The partitioned Bayesian phylogenetic tree based on *ND4* haplotypes for *E. argus*. Nodes N0 to N12 denote internal nodes of interest. Numbers above the branches are Bayesian posterior probabilities. Taxa are haplotypes; all haplotype designations are listed in Appendix C, followed by the sampling localities and numbers of individuals from each locality having that haplotype [e.g., JY(1)].

data, and Harpending's raggedness Index (Fu, 1997; Excoffier *et al.*, 2005). To further estimate past population dynamics through time, we constructed BSP implemented in BEAST 1.6.1 (Drummond and Rambaut, 2007) with a relaxed molecular clock method to depict the change of the two species' effective population size since the time to the most recent common ancestor (TMRCA) of the sampled *ND4* haplotypes respectively. For each BSP, the model of molecular evolution was assessed for the *ND4* sequence by AIC in MrModeltest 2.3 (Nylander, 2004).

We selected the best-fit models (GTR + G) and (GTR + I + G) for *E. argus* and *E. brenchleyi*, respectively. The only one calibration point based on the fossil record from the Middle Pleistocene (0.438–0.548 Ma) (Li *et al.*, 2004) was assigned to the most recent common ancestor (MRCA) of *E. argus* and for this species we assumed a normal prior distribution centered at 0.493 Ma with a standard deviation of 0.028 Ma for the MRCA. For *E. brenchleyi*, the evolutionary rate of *ND4* was set to the ucd.mean of BSP of *E. argus* above because no fossil

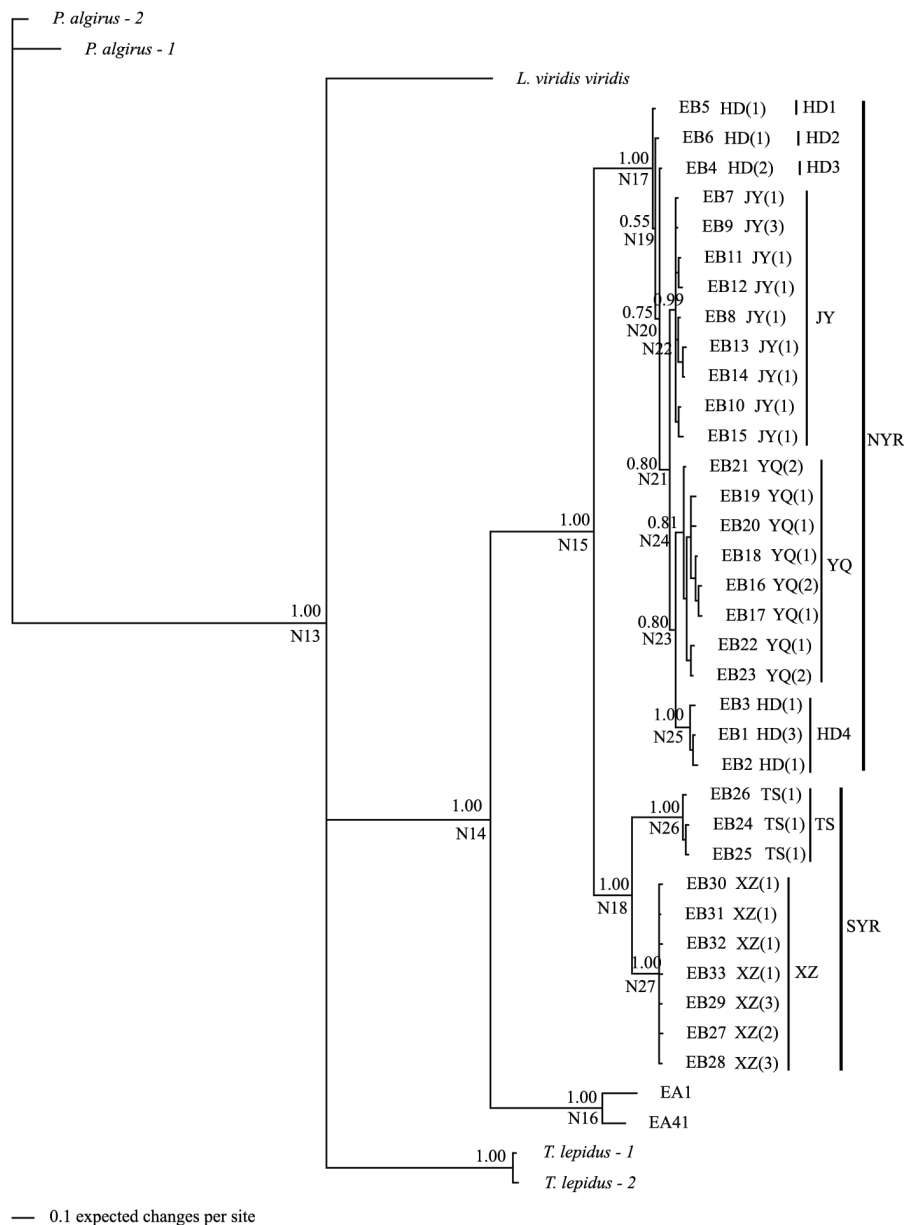


Figure 3 The partitioned Bayesian phylogenetic tree based on *ND4* haplotypes for *E. brenchleyi*. Nodes from N13 to N27 denote internal nodes of interest. Numbers above the branches are Bayesian posterior probabilities. Taxa are haplotypes; all haplotype designations are listed in Appendix C, followed by the sampling localities and numbers of individuals from each locality having that haplotype [e.g., JY(1)].

calibration points are available. MCMC sampling was run for 3×10^7 generations, with a sample frequency of 100 after a burn-in of 3×10^5 generations. The effective sample size of each parameter was measured and demographic plots for the two species were visualized in Tracer version 1.5 (Rambaut and Drummond, 2007). We also made the diagram of oxygen isotopic ($\delta^{18}\text{O}$) record during the past 0.78 Ma as proxy for global ice distribution for each species (Imbrie *et al.*, 1984).

We computed the pairwise Φ_{ST} value for each combination of populations using the Kimura two-

parameter model of nucleotide substitution (Kimura, 1981), which takes into account haplotype frequencies and the genetic distance between haplotypes in ARLEQUIN. The significance of Φ_{ST} values was evaluated using 1000 permutations of the data and set at $P = 0.05$. We performed a Mantel test to compare the pairwise population structure estimates to a matrix of geographical distance. We used ARLEQUIN also to perform a two-level hierarchical analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) to test for structure among populations: within populations, and

among populations (Φ_{ST}).

One additional procedure was used to characterize major patterns of genetic structure across the ranges sampled for both species. We applied the SAMOVA procedure (Dupanloup *et al.*, 2002) to identify groups of collection localities that were maximally differentiated based on *ND4*, and to infer genetic barriers between these groups. We used SAMOVA 1.0 to perform analyses based on 100 simulated annealing steps and examined the number of groups (k) that maximized genetic differentiation among groups (Φ_{CT}). The analysis was run for $k = 2$ to $k = n$ ($n = 8$ for *E. argus*; $n = 4$ for *E. brenchleyi*) and the significance level of fixation indices was evaluated by 1000 permutations.

3. Results

3.1 Matrilineal patterns We obtained 44 *E. argus* haplotypes (Appendix A) and 33 *E. brenchleyi* haplotypes (Appendix B). The ratio of haplotypes relative to the total number of individuals sampled was 0.34 for *E. argus* and 0.71 for *E. brenchleyi*. Neither mean nucleotide diversity ($F_{1,12} = 0.69$, $P = 0.42$) nor the number of haplotypes ($F_{1,12} = 0.96$, $P = 0.35$) per sampling location differed between the two species (Table 1). The number of haplotypes, the number of unique haplotypes, haplotype diversity and nucleotide diversity for single sampling localities were not correlated with latitude in either species (all $P > 0.26$). All *E. argus* haplotypes formed a single well-supported clade, which could be subdivided into three clades (EA I, EA II and EA III) although the phylogenetic relationships among these clades were not fully resolved ($PP < 0.50$) (Figure 2). Clade EA I included a single haplotype (EA44) from the Chuzhou (CZ) population. Clade EA II was poorly supported ($PP = 0.66$) and further subdivided into five clades: one poorly supported clade (EA Ila, $PP = 0.63$) and four well-supported clades (EA I Ib, $PP = 1.00$; EA I Ic, $PP = 0.98$; EA I Id, $PP = 1.00$; EA I Ie, $PP = 1.00$). These five clades were mostly admixed in many local populations and had no geographical specificity. Clade EA I Ia was further subdivided into two poorly supported clades (EA I Ia1, $PP = 0.71$; EA I Ia2, $PP = 0.88$) that were mostly admixed in the Handan (HD) population and had no geographical specificity. Clade EA III included two haplotypes from the Harbin (HRB) population.

All *E. brenchleyi* haplotypes formed a single well-supported clade, which could be subdivided into two well-supported clades [north (NYR) and south (SYR) of the Yellow River, both $PP = 1.00$] (Figure 3). The NYR clade included all haplotypes from the Yangquan (YQ),

HD and Jiyan (JY) populations in NYR, and the SYR clade included all haplotypes from the Xuzhou (XZ) and Taishan (TS) populations in SYR. The division of the two clades coincided with the Yellow River (Figure 1). There was a clear population-genetic structure within both clades. The NYR clade could be subdivided into three major clades (HD4, $PP = 1.00$; JY, $PP = 0.99$; YQ, $PP = 0.81$) and three minor clades (HD1, HD2 and HD3) of which each included a single haplotype from the HD population east of the Taihang Mts (36° – 40° N, 112° – 115° E). The HD4 clade included haplotypes all from the HD population; the JY clade included all haplotypes from the JY population also in east of the Taihang Mts; the YQ clade included all haplotypes from the YQ population west of the Taihang Mts. The HD1, HD2, HD3, HD4 and JY clades formed one group including all haplotypes from two local populations (HD and JY) east of the Taihang Mts; the YQ clade formed the other group including all haplotypes from the YQ population west of the Taihang Mts. The geographical division of these two groups coincided with the Taihang Mts (Figure 1). The SYR clade could be subdivided into two clades with strong nodal support (TS clade and XZ clade, both $PP = 1.00$). The TS clade included all haplotypes from the TS population, while the XZ clade included all haplotypes from the XZ population.

For *E. argus*, results of the Median-joining network (MJN) suggests little or no association between haplotypes and geography (Figure 4 A, Appendix A). We detected related haplotypes that formed eight groups (EA I, EA I Ia1, EA I Ia2, EA I Ib, EA I Ic, EA I Id, EA I Ie and EA III) in the Bayesian phylogenetic tree (Figure 2, Figure 4 A). Clade EA I Ia2 and EA I Ic co-occurred in Xinghe (XH) and YQ. Clade EA I Ia1 and EA I Ib co-occurred in HD, JY and Chang'an (CA). Clade EA I Ia1 and EA I Id co-occurred in HRB and CA. Clade EA I Ia1, EA I Ib, EA I Id and EA I Ie co-occurred in CA. Clade EA I Ia1, EA I Ic, EA I Id and EA III co-occurred in HRB. For *E. brenchleyi*, however, we detected networks of related haplotypes that were clustered into two distinct areas (SYR and NYR) respectively corresponding to the SYR clade and NYR clade in the Bayesian phylogenetic tree (Figure 3, Figure 4 B, Appendix B). We further detected networks of related SYR haplotypes that were clustered into two distinct areas (TS and XZ), and detected networks of related NYR haplotypes that were clustered into two distinct areas [YQ and (HD1, HD2, HD3, HD4, and JY)]. These four areas [(HD1, HD2, HD3, HD4, and JY), YQ, TS, and XZ] correspond to clades HD1, HD2, HD3, HD4, and JY, clade YQ, clade TS, and clade XZ,

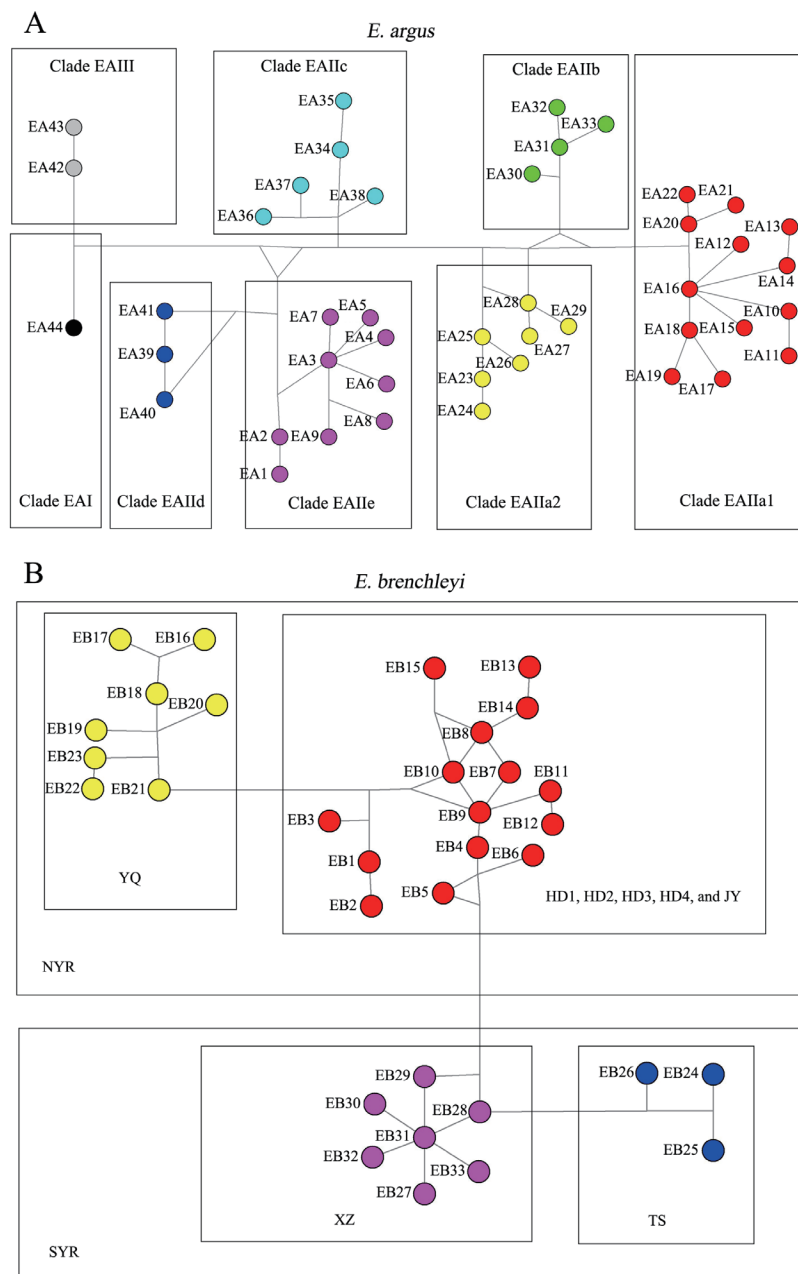


Figure 4 Median-joining networks of mtDNA haplotypes at the *ND4* gene in *E. argus* (A) and *E. brenchleyi* (B). Numbers adjacent to open circles (network nodes) refer to haplotypes listed in Appendices A and B. Haplotype group color for *E. argus* is consistent with Figure 1 A, and haplotype group color for *E. brenchleyi* is consistent with Figure 1 B.

respectively (Figure 3).

3.2 Divergence time Table 2 gives posterior distributions of divergence time at each node and 95% credibility intervals based on phylogenies. For *E. argus*, the most recent common ancestor (TMRCA) of clade EA IIa1 (node N7), EA IIa2 (node N8), EA IIb (node N9), EA IIc (node N10), EA IId (node N11), EA IIE (node N12), and EA III (node N5) were estimated at 0.158 ± 0.063 , 0.178 ± 0.064 , 0.205 ± 0.087 , 0.220 ± 0.082 , 0.144 ± 0.082 ,

0.184 ± 0.082 and 0.100 ± 0.067 Ma, respectively. For *E. brenchleyi*, the divergence time of the NYR clade from the SYR clade (node N15) was dated to 3.208 ± 0.743 Ma. Within the NYR clade, the divergence time of clades HD1, HD2, HD3, HD4 and JY from clade YQ (node N23) was dated to 0.939 ± 0.418 Ma. Within the SYR clade, the divergence time of the TS clade from the XZ clade (node N18) was dated to 2.095 ± 0.703 Ma. The TMRCA of clades JY and HD (node N17) was estimated at 1.798

Table 2 The posterior distribution of divergence times with 95% confidence intervals estimated by Bayesian molecular dating. DT: divergence times (Ma); SD: standard deviation; CI: 95% credibility interval.

Species	Node	DT	SD for DT	CI for DT
<i>E. argus</i>	N0	9.195	3.13	4.420–16.562
	N1	4.809	1.427	2.605–8.141
	N2	2.292	0.878	0.976–4.367
	N3	0.498	0.031	0.441–0.546
	N4	0.376	0.076	0.225–0.512
	N5	0.1	0.067	0.006–0.256
	N6	0.255	0.077	0.122–0.420
	N7	0.158	0.063	0.056–0.303
	N8	0.178	0.064	0.073–0.322
	N9	0.205	0.087	0.056–0.391
	N10	0.22	0.082	0.074–0.395
	N11	0.144	0.082	0.024–0.336
<i>E. brenchleyi</i>	N12	0.184	0.082	0.055–0.372
	N13	5.057	1.482	2.646–8.532
	N14	3.86	0.87	2.163–5.561
	N15	3.208	0.743	1.648–4.309
	N16	0.495	0.032	0.441–0.545
	N17	1.798	0.615	0.700–3.035
	N18	2.095	0.703	0.817–3.482
	N19	1.504	0.55	0.539–2.647
	N20	1.29	0.504	0.442–2.378
	N21	1.1	0.461	0.354–2.117
	N22	0.735	0.394	0.167–1.666
	N23	0.939	0.418	0.283–1.884
	N24	0.757	0.369	0.207–1.622
	N25	0.575	0.355	0.089–1.429
	N26	0.404	0.324	0.044–1.251
	N27	0.262	0.264	0.012–0.996

± 0.615 Ma; the TMRCA of clades YQ (node N24), XZ (node N27) and TS (node N26) were estimated at 0.757 ± 0.369 , 0.262 ± 0.264 and 0.404 ± 0.324 Ma, respectively.

3.3 Population-genetic analysis For *E. argus*, mismatch distributions of the Gonghe (GH) and CZ populations exhibit relatively unimodal patterns with non-significant raggedness values, matching the expected distribution of an expanding population well, and supporting that these two populations have undergone rapid demographic expansion. Mismatch distributions of the remaining seven populations show multimodal distributions. Significant *P*-values for the sum of squares deviations (SSD), high raggedness indexes, positive Fu's F_s and Tajima's *D* values indicate no expansion in the YQ and HD populations, and negative Tajima's *D* values suggest recent expansion in the HRB, XH, Etuohe (ETK), JY and CA populations.

For *E. brenchleyi*, mismatch distributions of the TS, JY and XZ populations exhibit relatively unimodal patterns

with on-significant raggedness values, fitting the expected distribution of an expanding population (non-significant SSD) and supporting that these three populations have undergone rapid demographic expansion. Mismatch distributions of the YQ and HD populations show multimodal distributions. The significant *P*-value for SSD, high raggedness index, positive Fu's F_s and Tajima's *D* values indicate no population expansion in the HD population, and negative Fu's F_s value, non-significant SSD and low raggedness index indicate recent population expansion in the YQ population.

For all populations of *E. argus*, the multimodal mismatch distribution, non-significant *P*-value for SSD, low but significant raggedness index and negative Fu's F_s and Tajima's *D* values indicate these populations as a whole have possibly undergone recent expansion. The Bayesian skyline plot of *E. argus* suggested that population size remained relatively stable or grew slowly through time during both cold (positive $\delta^{18}\text{O}$ values) and warm periods (negative $\delta^{18}\text{O}$ values) (Figure 5). Increase was followed by a decline starting at about 0.0496 Ma, and then decline was followed by an increase starting at 0.004 39 Ma.

For all populations of *E. brenchleyi*, negative Fu's F_s values indicate populations as a whole have possibly undergone recent expansion. The mismatch distribution showed multimodal, and the *P*-value for SSD and the raggedness index with low-value both were non-significant. To construct Bayesian skyline plots (BSP) of *E. brenchleyi*, the rate of evolution of *ND4* was set to 0.064 5 substitutions per site per Ma (from the ucl. mean of BSP of *E. argus*). The Bayesian skyline plot of *E. brenchleyi* implied that population size remained relatively stable or descend slowly through time during both cold (positive $\delta^{18}\text{O}$ values) and warm periods (negative $\delta^{18}\text{O}$ values) (Figure 6). Decline was followed by an increase starting at about 0.030 6 Ma. The TMRCA of *E. argus* and *E. brenchleyi* were estimated about 0.493 Ma (with the 95% credibility interval ranging from 0.438 to 0.548 Ma) and 1.514 Ma (with the 95% credibility interval ranging from 0.922 to 2.248 Ma), respectively.

Results of AMOVA indicated the presence of geographic genetic structure within both species, concordant with results of each BSP above. For *E. argus*, a two-level AMOVA to test for structure among populations detected high genetic structure ($\Phi_{ST} = 0.594$, $P < 0.001$). A small portion of the total genetic structure exists within populations, and the majority was among populations ($\Phi_{ST} = 0.594$, $P < 0.001$). For *E. brenchleyi*, a two-level AMOVA to test for structure among populations

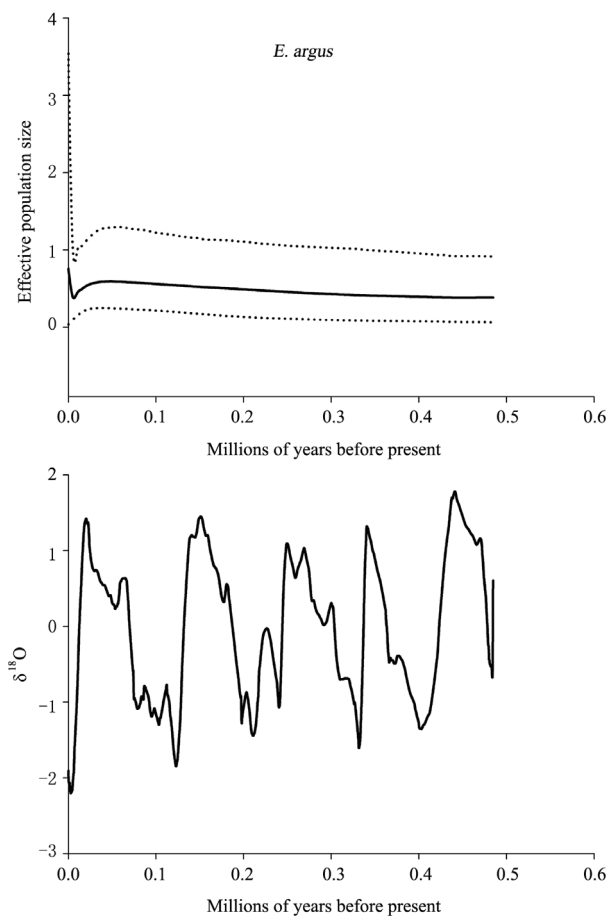


Figure 5 Pleistocene ice distribution (bottom) and Bayesian skyline plot (BSP) (top) representing the historical demographic trend of mitochondrial lineages of *E. argus*. The solid line represents the mean of the effective population size, while dotted lines represent the 95% HPD limits. The $\delta^{18}\text{O}$ curve is used as proxy for global ice distribution (data from the SPECMAP project: <http://www.ngdc.noaa.gov/mgg/geology/specmap.html>). Negative values represent less ice; positive values represent more extensive ice coverage.

detected relatively higher genetic structure ($\Phi_{\text{ST}} = 0.908$, $P < 0.001$). A small portion of the total genetic structure exists within populations, and the majority was among populations ($\Phi_{\text{ST}} = 0.905$, $P < 0.001$).

For *E. argus*, pairwise area estimates of Φ_{ST} ranged from 0.149 to 0.868. All of the 36 pairwise Φ_{ST} values were statistically significant (Table 3). Results of the Mantel test between Φ_{ST} and geographical distance showed a weak relationship between population structure and distance ($R^2 = 0.18$, $P = 0.096$). For *E. brenchleyi*, pairwise area estimates of Φ_{ST} ranged from 0.525 to 0.972. All of the 10 pairwise Φ_{ST} values were significant (Table 4). The results of the Mantel test between Φ_{ST} and geographical distance showed a significant relationship between population structure and distance ($R^2 = 0.43$, $P = 0.007$). Thus, only *E. brenchleyi* shows conclusive

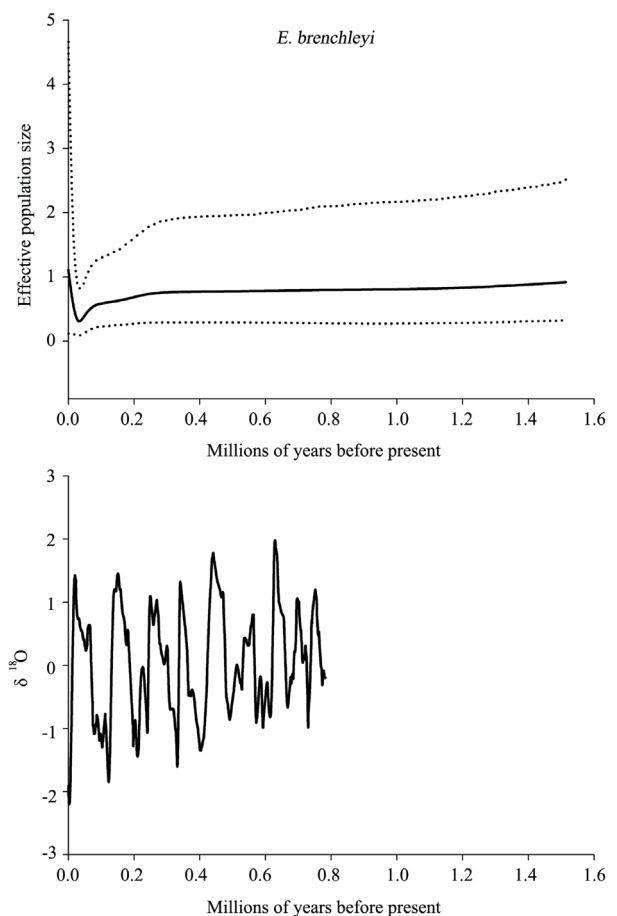


Figure 6 Pleistocene ice distribution (bottom) and Bayesian skyline plot (top) representing the historical demographic trend of mitochondrial lineages of *E. brenchleyi*. The solid line represents the mean of the effective population size, while dotted lines represent the 95% HPD limits. The oxygen isotopic curve ($\delta^{18}\text{O}$) is plotted as proxy for global ice distribution (data from the SPECMAP project: <http://www.ngdc.noaa.gov/mgg/geology/specmap.html>). Negative values represent less extensive ice coverage; positive values represent more extensive ice coverage.

evidence of isolation by distance.

Table 5 shows results of SAMOVA. For *E. argus* group structure was maximized at $k = 7$ ($\Phi_{\text{CT}} = 0.504$). The maximum among-group structure was: JY and CA vs. HRB vs. XH and YQ vs. GH vs. ETK vs. HD vs. CZ. For *E. brenchleyi*, there were distinct groups of genetically defined sampling areas, with Φ_{CT} ranging from 0.489 to 0.672 when the program was instructed to identify $k = 2$ through $k = 4$. Partitions of sampling areas were identified that suggested SYR vs. NYR groups (partitions: YQ, HD and JY vs. TS and XZ; $\Phi_{\text{CT}} = 0.708$) at $k = 2$, and this grouping pattern was concordant with the division of the Yellow River and Bayesian phylogenetic tree (Figure 3). Group structure was maximized at $k = 3$ ($\Phi_{\text{CT}} = 0.808$). However, partitions of sampling areas suggest YQ vs. HD

Table 3 *Eremias argus* pairwise estimates of genetic structure (Φ_{ST}), + represents significance at $P < 0.05$.

	HRB	XH	ETK	YQ	HD	GH	JY	CA	CZ
HRB	0								
XH	0.702+	0							
ETK	0.810+	0.835+	0						
YQ	0.673+	0.334+	0.786+	0					
HD	0.614+	0.377+	0.732+	0.407+	0				
GH	0.726+	0.798+	0.868+	0.598+	0.628+	0			
JY	0.666+	0.582+	0.792+	0.539+	0.285+	0.767+	0		
CA	0.521+	0.382+	0.636+	0.426+	0.229+	0.545+	0.149+	0	
CZ	0.556+	0.489+	0.706+	0.470+	0.329+	0.604+	0.369+	0.310+	0

Table 4 *Eremias brenchleyi* pairwise estimates of genetic structure (Φ_{ST}), + represents significance at $P < 0.05$.

	YQ	HD	TS	JY	XZ
YQ	0				
HD	0.525+	0			
TS	0.940+	0.879+	0		
JY	0.723+	0.531+	0.970+	0	
XZ	0.956+	0.922+	0.960+	0.972+	0

and JY vs. TS vs. XZ [respectively corresponding to clade YQ, (clades HD1, HD2, HD3, HD4 and JY), clade TS and clade XZ in the Bayesian phylogenetic tree (Figure 3)] at $k = 4$, with only a minimal reduction in group structure ($\Phi_{CT} = 0.769$).

4. Discussion

4.1 Phylogeographical patterns Bayesian analysis indicated that *E. argus* was composed of eight matrilineages and had no geographical specificity. Unlike *E. argus*, there were two reciprocally well-supported lineages (NYR and SYR) within *E. brenchleyi*, and their geographical separation occurred at the Yellow River (Figure 1, Figure 3). Results of MJN analysis revealed geographic genetic structuring in the two species, coinciding with results of the Bayesian analysis. There are no evident signs of isolation in *E. argus*, but the Yellow River and Taihang Mts may have acted as important barriers to gene flow in *E. brenchleyi*. SAMOVA analysis of *E. brenchleyi* indicated that overall haplotypes were clustered into four distinct areas, corresponding to the four phylogeographical units (Avise, 2000) and concordant with the Bayesian and MJN analyses.

The Yellow River formed during the initial stage of the early Pleistocene (about 1.6 Ma) (Li *et al.*, 1996; Zhang *et al.*, 2003), and the divergence time between the two clades in *E. brenchleyi* was estimated at 3.208 ± 0.743 Ma (with the 95% credibility interval ranging from 1.648 to 4.309 Ma). Accordingly, we conclude that *E.*

brenchleyi may have been separated by the Yellow River into two lineages (NYR and SYR). This conclusion is consistent with that drawn previously using *cyt b* gene (Zhao *et al.*, 2011). Within clade NYR, the divergence time of the (HD1+HD2+HD3+HD4+JY) group from the YQ group was dated to 0.939 ± 0.418 Ma (with the 95% credibility interval ranging from 0.283–1.884 Ma). Therefore, the NYR lineage may have been separated by the Taihang Mts into two groups (Figure 1, Figure 3) because Taihang Mts was uplifted to 1100–1500 m during the Pleistocene-Holocene (Wu *et al.*, 1999). The SYR lineage may have been separated into two groups (TS and CZ), although we did not detect the mountain or river with which the geographical division of the clades TS and CZ coincided. The mantel test revealed that *E. brenchleyi* had more strongly restricted gene flow among populations than did *E. argus*. We suppose that genetic divergence results primarily from the lack of gene flow between the two regions (TS and CZ), probably because they are far enough from each other to result in isolation by distance. Dispersal ability is greater in *E. argus* than in *E. brenchleyi* (Zhao *et al.*, 2011), so the Yellow River may be less likely to serve as a barrier for *E. argus* to disperse between SYR and NYR during Yellow River zero-flow, and the Taihang Mts are also less likely to act as a boundary between the lineages of *E. argus*. This conclusion is consistent with that drawn for *E. argus* based on *cyt b* gene data (Zhao *et al.*, 2011).

AMOVA analysis indicated that among-population structure was high in *E. argus* and even higher in *E. brenchleyi*, consistent with many studies showing that geographic genetic structuring is strong for mtDNA variation in lizards (Clark *et al.*, 1999; Stenson and Thorpe, 2003; Jin *et al.*, 2008; Urquhart *et al.*, 2009). The range of genetic structure is narrower in *E. argus* (from 0.149 to 0.868) than in *E. brenchleyi* (from 0.525 to 0.972). *Eremias brenchleyi* had more significant geographical structuring of haplotype diversity than *E. argus*.

Table 5 Result of SAMOVA using mitochondrial *ND4* sequences. The best number of groups has the largest Φ_{CT} value

Species	Groups	Group composition	Among groups	Among populations within groups	Among populations	Percentage of variation (%)		
			Φ_{CT}	Φ_{SC}	Φ_{ST}	Among groups	Among populations within groups	Within population
<i>E. argus</i>	Two groups	ETK vs. HRB, XH, YQ, HD, GH, JY, CA, and CZ	0.501	0.519***	0.76***	50.13	25.89	23.98
	Three groups	XH, YQ, HD, GH, JY, CA, and CZ vs. HRB vs. ETK	0.436*	0.445***	0.687***	43.56	25.1	31.34
	Four groups	HD, JY, CA, and CZ vs. XH, YQ, and GH vs. ETK vs. HRB	0.432***	0.356***	0.634***	43.16	20.22	36.62
	Five groups	HD, JY, and CA vs. HRB vs. ETK vs. CZ vs. XH, YQ, and GH	0.449**	0.311***	0.62***	44.89	17.14	37.97
	Six groups	ETK vs. XH and YQ vs. HD, JY, and CA vs. HRB vs. CZ vs. GH	0.484***	0.253***	0.615***	48.38	13.08	38.55
	Seven groups	JY and CA vs. HRB vs. XH and YQ vs. GH vs. ETK vs. HD vs. CZ	0.504***	0.203***	0.604***	50.39	10.05	39.55
	Eight groups	XH vs. ETK vs. YQ vs. HD vs. GH vs. CZ vs. JY and CA vs. HRB	0.499*	0.202***	0.6***	49.89	10.14	39.97
<i>E. brenchleyi</i>	Two groups	YQ, HD, and JY vs. TS and XZ	0.708	0.766***	0.932***	70.84	22.35	6.81
	Three groups	TS vs. YQ, HD, and JY vs. XZ	0.808	0.65***	0.933***	80.77	12.49	6.74
	Four groups	YQ vs. HD and JY vs. TS vs. XZ	0.769	0.615***	0.911***	76.87	14.22	8.91

* indicates significance at $P < 0.05$; ** indicates significance at $P < 0.01$; *** indicates significance at $P < 0.001$.

4.2 Genetic diversity and multiple refugia Our data show that neither in *E. argus* nor *E. brenchleyi* are diversity indices correlated with latitude and that genetic diversity is almost homogeneous across the range of each species. These findings are consistent with *cyt b* data reported previously for the two species (Zhao *et al.*, 2011) but not with phylogeographic studies of widespread terrestrial species in North America and Europe, where they expanded from southern refugia at the end of the Pleistocene, thus exhibiting more geographically structured genetic diversity in the south (Hewitt, 1996, 1999, 2000). It may be the case that multiple refugia were maintained across the range of each species during the LGM, with neither species expanding from a single refuge.

Bayesian analysis shows that the haplotype clade of *E. argus* is composed of six major clades (EAIa1, EAIa2, EAIb, EAIc, EAIId and EAIe) with strong nodal support (all PP > 0.7). The TMRCA of these six clades is estimated to be 0.158 ± 0.063 , 0.178 ± 0.064 , 0.205 ± 0.087 , 0.220 ± 0.082 , 0.144 ± 0.082 and 0.184 ± 0.082 Ma, respectively. The haplotype clade of *E. brenchleyi* is composed of five major clades (JY, YQ, HD4, TS and XZ) with strong nodal support (all PP > 0.8). The TMRCA of these five clades is estimated to be about 0.735 ± 0.394 , 0.757 ± 0.369 , 0.575 ± 0.355 , 0.404 ± 0.324 and 0.262 ± 0.264 Ma, respectively. Thus, all major clades for these two lizards formed before the LGM (about 0.06–0.1 Ma) (Tian *et al.*, 2009). Considering that ancestral haplotypes

are more likely to be found at refugia (Slatkin, 1996; Parsons *et al.*, 1997; Li *et al.*, 2009), and the TMRCA of these clades, it is possible that six major clades of *E. argus* and five major clades of *E. brenchleyi* diagnose six separate glacial refugia in *E. argus* and five separate glacial refugia in *E. brenchleyi* during the LGM.

Furthermore, we propose an alternative hypothesis that each species expanded from a single refuge after the LGM. In this case, related haplotypes could be expected to form a star-like network circling the ancestral haplotype (Avise, 2000), and among-population structure could be expected to be low (Hewitt, 2000). In contrast to these expectations, we detected a reticulated rather than star-like network in both species (Figure 4), and found high among-population structure in *E. argus* ($\Phi_{ST} = 0.594$, $P < 0.001$) and even higher among-population structure in *E. brenchleyi* ($\Phi_{ST} = 0.908$, $P < 0.001$) that probably resulted from genetic drift favouring/fixing different alleles among multiple independent refugia during the LGM (Tian *et al.*, 2009). Multiple isolated refugia promote genetic differentiation between current populations (Avise, 2000). Our findings support the existence of multiple refugia in both *E. argus* and *E. brenchleyi*, and are consistent with the results reported in similar phylogeographic studies (Hewitt, 1996, 2000; Zamudio and Savage, 2003; Li *et al.*, 2009; Tian *et al.*, 2009). Multiple glacial refugia may have been available to East Asian species throughout their entire ranges (Li *et al.*, 2009), probably because East Asia potentially hosts microclimatic zones capable of

supporting a variety of habitats in relative stability (Qian and Ricklefs, 2000).

4.3 Demographic history Populations of *E. argus* and *E. brenchleyi* showed genetic imprints of demographic expansion as inferred by a negative *F_s* test, which was also supported by the multimodal mismatch distributions analysis, Rogers test of sudden expansion model and AMOVA (Rogers *et al.*, 1996; Castoe *et al.*, 2007; Nuñez *et al.*, 2011). According to the inference of population expansion, *E. argus* has undergone historical slow growth or is stable in size and *E. brenchleyi* has undergone historical slow decline in size.

All populations of *E. argus* remained relatively stable or grew slowly during cold (positive $\delta^{18}\text{O}$ values) and warm (negative $\delta^{18}\text{O}$ values) periods, probably because the species survived in multiple refugia during cold periods (stadials). Occasional migration among expanded populations might have resumed during warmer periods (interstadials), and this intermittent gene flow between refugia might have made the species population size insensitive to population reduction resulting from cool climates (Li *et al.*, 2009).

All populations of *E. brenchleyi* remained relatively stable or descended slowly through time during cold and warm periods, probably because there had been lower intermittent gene flow among populations in *E. brenchleyi* than in *E. argus*. Moreover, the expansion events beginning at 0.004 39 Ma for *E. argus* and 0.030 6 Ma for *E. brenchleyi* suggest that climate changes during the LGM may have differentially affected the demographic histories of these two species.

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Supplemental Materials

Appendix A *Eremias argus* haplotype frequency by population. Haplotype labels follow Figure 2 and Figure 4 A.

Haplotype number	HRB	XH	ETK	YQ	HD	GH	JY	CA	CZ
EA1								1	
EA2								2	
EA3			4						
EA4			1						
EA5			1						
EA6			1						
EA7			1						
EA8			1						
EA9			1						
EA10	1							15	
EA11								1	
EA12							1		
EA13								5	
EA14								1	
EA15							2		
EA16							2		
EA17							2		
EA18							3		
EA19							1		
EA20					3				
EA21					1				
EA22					1				
EA23		1		4					
EA24				1					
EA25				1					
EA26				1					
EA27					1				
EA28		5							
EA29		5							
EA30								2	
EA31					3				
EA32							1		
EA33					2				
EA34	1					6			
EA35		1				6			
EA36				4					
EA37				1					
EA38			1						
EA39	2								
EA40	1								
EA41	15							2	
EA42	1								
EA43	1								
EA44									7

Appendix B *Eremias brenchleyi* haplotype frequency by population. Haplotype labels follow Figure 3 and Figure 4 B.

Haplotype number	YQ	HD	TS	JY	XZ
EB1		3			
EB2		1			
EB3		1			
EB4		2			
EB5		1			
EB6		1			
EB7				1	
EB8				1	
EB9				3	
EB10				1	
EB11				1	
EB12				1	
EB13				1	
EB14				1	
EB15				1	
EB16	2				
EB17	1				
EB18	1				
EB19	1				
EB20	1				
EB21	2				
EB22	1				
EB23	2				
EB24			1		
EB25			1		
EB26			1		
EB27					2
EB28					3
EB29					3
EB30					1
EB31					1
EB32					1
EB33					1

Appendix C GenBank accession numbers for the two lizard species in this study. GAN: GenBank accession number.

Haplotype number	GAN	Haplotype number	GAN
EA1	JN561171	EB1	JN561215
EA2	JN561172	EB2	JN561216
EA3	JN561173	EB3	JN561217
EA4	JN561174	EB4	JN561218
EA5	JN561175	EB5	JN561219
EA6	JN561176	EB6	JN561220
EA7	JN561177	EB7	JN561221
EA8	JN561178	EB8	JN561222
EA9	JN561179	EB9	JN561223
EA10	JN561180	EB10	JN561224
EA11	JN561181	EB11	JN561225
EA12	JN561182	EB12	JN561226
EA13	JN561183	EB13	JN561227
EA14	JN561184	EB14	JN561228
EA15	JN561185	EB15	JN561229
EA16	JN561186	EB16	JN561230
EA17	JN561187	EB17	JN561231
EA18	JN561188	EB18	JN561232
EA19	JN561189	EB19	JN561233
EA20	JN561190	EB20	JN561234
EA21	JN561191	EB21	JN561235
EA22	JN561192	EB22	JN561236
EA23	JN561193	EB23	JN561237
EA24	JN561194	EB24	JN561238
EA25	JN561195	EB25	JN561239
EA26	JN561196	EB26	JN561240
EA27	JN561197	EB27	JN561241
EA28	JN561198	EB28	JN561242
EA29	JN561199	EB29	JN561243
EA30	JN561200	EB30	JN561244
EA31	JN561201	EB31	JN561245
EA32	JN561202	EB32	JN561246
EA33	JN561203	EB33	JN561247
EA34	JN561204		
EA35	JN561205		
EA36	JN561206		
EA37	JN561207		
EA38	JN561208		
EA39	JN561209		
EA40	JN561210		
EA41	JN561211		
EA42	JN561212		
EA43	JN561213		
EA44	JN561214		